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14. ABSTRACT This proposal addressed genetic events that take place in the affected bone of sporadic Paget's disease of bone (PDB) patients. Microdissection revealed somatic SQSTM1 mutations in a subset of osteoblasts in pagetic bone. This suggests that pagetic lesions originate in this subset of cells. Our hypothesis is that sporadic PDB arises from a combination of somatic mutations and environmental events and these mutant-containing cells then recruit normal bone cells to the abnormal bone formation. This subset of mutation-containing cells also affects gene expression throughout the pagetic lesion. To test this, we proposed two specific aims: 1) to characterize this subset of cells within the pagetic bone. 2) to determine whether a subset of mutation-containing cells can affect the overall culture containing cells with and without mutation. Our study found that genes involved in signaling between osteoblasts and osteoclasts as well as the Toll-like receptor and Hedgehog signaling pathways were upregulated in SQSTM1 mutant positive pagetic bone supporting our model whereby SQSTM1 mutations in the osteoblasts initiate PDB. This is a fundamentally novel paradigm for SQSTM1-mediated PDB, which places the osteoblast squarely in the center of the action. It opens new PDB research and new targets for therapeutic intervention.					
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Introduction

This proposal addressed the FY10 PRMRP topic of Paget’s disease of bone (PDB). This proposal addressed the genetic events that place in the affected bone of PDB patients who do not have the familial form of the disease. Laser capture microdissection of osteoblasts within the affected bone revealed somatic *SQSTM1* mutations had taken place in some patients. Curiously, it appeared that the *SQSTM1* mutation was not present in all of the osteoblasts in the pagetic bone, but rather in a subset of cells. This discovery suggests that this subset of cells must be the origin of the pagetic lesion and the mutations must have taken place in the cells at the site of the pagetic bone. This proposal is intended to characterize the frequency and nature of this critical subset of cells and how they give rise to pagetic bone formation. Our hypothesis for this application is that sporadic PDB arises as the result of a combination of somatic mutations and environmental events that give rise to the pagetic lesion and that in sporadic PDB there is a small population of cells that are critical to the formation of the pagetic bone. These mutant-containing cells recruit normal bone to the affected site and drive the abnormal bone formation phenotype. Moreover, this subset of mutation-containing cells affects gene expression throughout the overall pagetic lesion. To test this hypothesis, we proposed to use a combination of in vitro experiments to complete the following specific aims: 1) to demonstrate the presence and nature of this subset of critical cells within the pagetic bone. 2) to determine whether a subset of cells containing the critical mutation can affect the overall nature of a culture containing cells with and without the mutations.

Research Accomplishments

From the Statement of Work:

Specific Aim 1: To demonstrate the presence and nature of the subset of critical cells within the pagetic bone

Laser Capture Microdissection – we were able to capture homogeneous populations of cells from samples of normal and pagetic bone and isolate RNA from these samples. As shown by the qRT-PCR results shown in Figure 1, we were able to capture homogeneous populations of osteoblast-like cells from both normal and pagetic bone. Moreover, we were able to show differential expression of a number of different genes as shown in Figures 2-4.

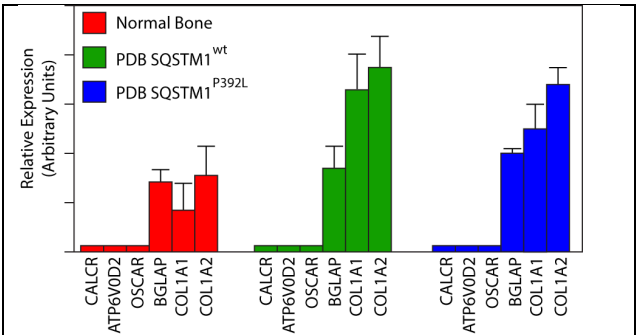


Figure 1. Relative expression of markers of OC and OB differentiation in the LCM captured PDB samples. Gene expression normalized to GAPDH and shown relative to each other within each sample.

Chemotactic signaling was upregulated and OPG was downregulated in *SQSTM1*^{P392L} pagetic bone: We found that

RANKL was upregulated in all PDB bone samples regardless of *SQSTM1* mutation status (Figure 3). This is consistent with previous reports that *SQSTM1*^{P392L} – carrying

cells express higher levels of *RANKL* than normal cells in response to stimulation by 1,25-(OH)₂D₃ (1). We also found that *OPG*, an inhibitor of *RANK* signaling, was downregulated in the *SQSTM1*^{P392L} mutant samples while *RANK* expression was slightly upregulated in the *SQSTM1*^{P392L} mutant samples (Figure 3). We were curious if other chemokines were upregulated in the *SQSTM1*^{P392L} bone samples. We found several other chemokines that were upregulated in the *SQSTM1*^{P392L} bone samples

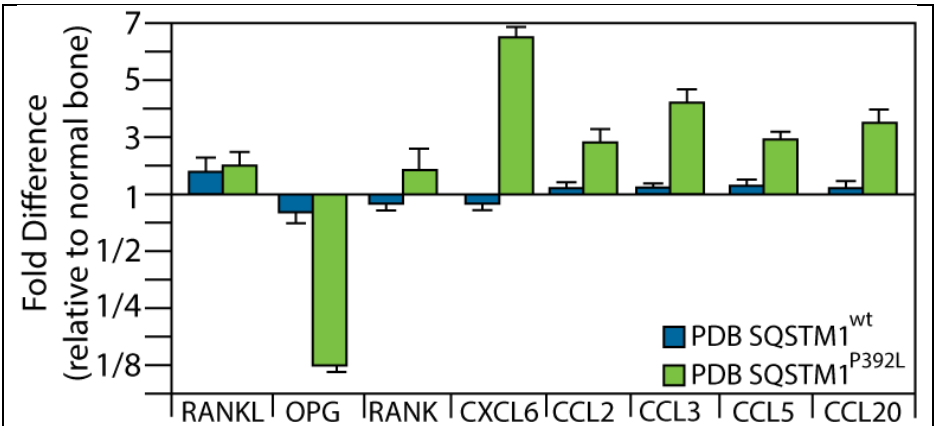


Figure 2. Expression of chemokine genes important in OB/OC signaling is elevated in PDB samples with *SQSTM1* mutations. Expression normalized to normal bone.

(Figure 2). These included known OC signaling molecules *CCL2* (MCP1), *CCL3* (MIP1A), *CCL5* (RANTES), *CCL20* (MIP3A) (2, 3). We also found upregulated *CXCL6*, a gene implicated in inflammation and wound healing (4).

Global expression analysis revealed upregulation of the TLR signaling pathway: We then analyzed the microarray expression data for the five pagetic bone samples and two normal bone samples using two software packages: Ingenuity Pathway Analysis (Ingenuity Systems; Redwood City, CA) and GeneSifter software package (Geospiza, Inc.; Seattle, WA) to detect altered signaling pathways. We were curious to know if there were specific signaling pathways that were altered in the samples with *SQSTM1* mutations and not in the normal bone or in the samples of PDB bone that were wildtype for *SQSTM1*. We found that the TLR signaling pathway appeared to be differentially activated in the *SQSTM1*^{mutant} samples (Figure 3). Specifically, elements of the MyD88-dependent pathway appear upregulated including receptors, *TLR1* and *TLR4* as well as the co-receptor *CD14*, *MyD88*, *IRAK4*, *TRAF6* had increased expression in the *SQSTM1*^{mutant} samples (Figure 3). Moreover, the downstream targets of the TLR signaling pathway including *TNFα*, *IL12*, *CCL2*, *IL8*, *CCL5*, *CCL3* and *IFNα* were all upregulated in expression in the *SQSTM1*^{mutant} samples (Figure 3) suggesting that *MyD88*-dependent TLR signaling was specifically activated in the *SQSTM1*^{mutant} samples.

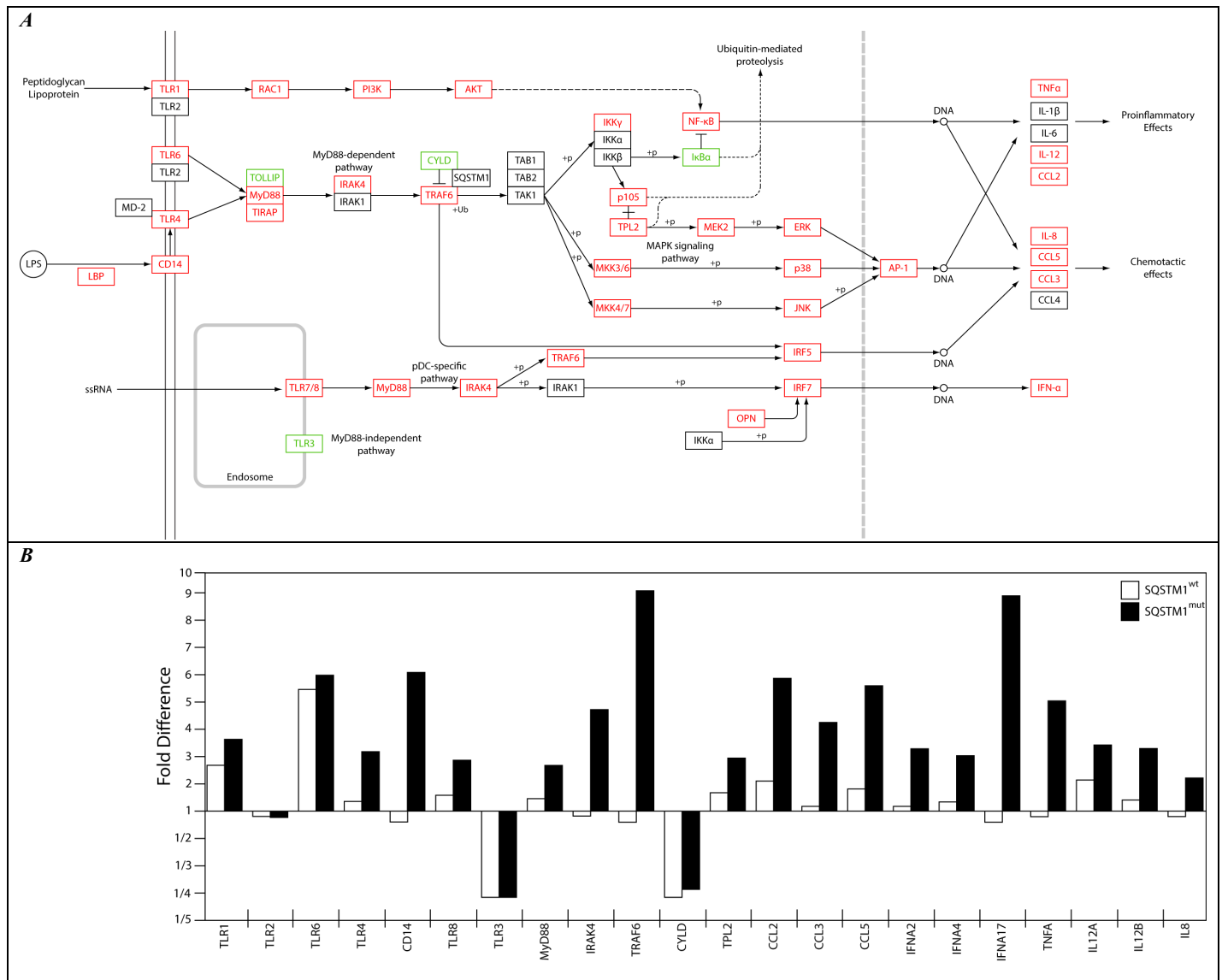
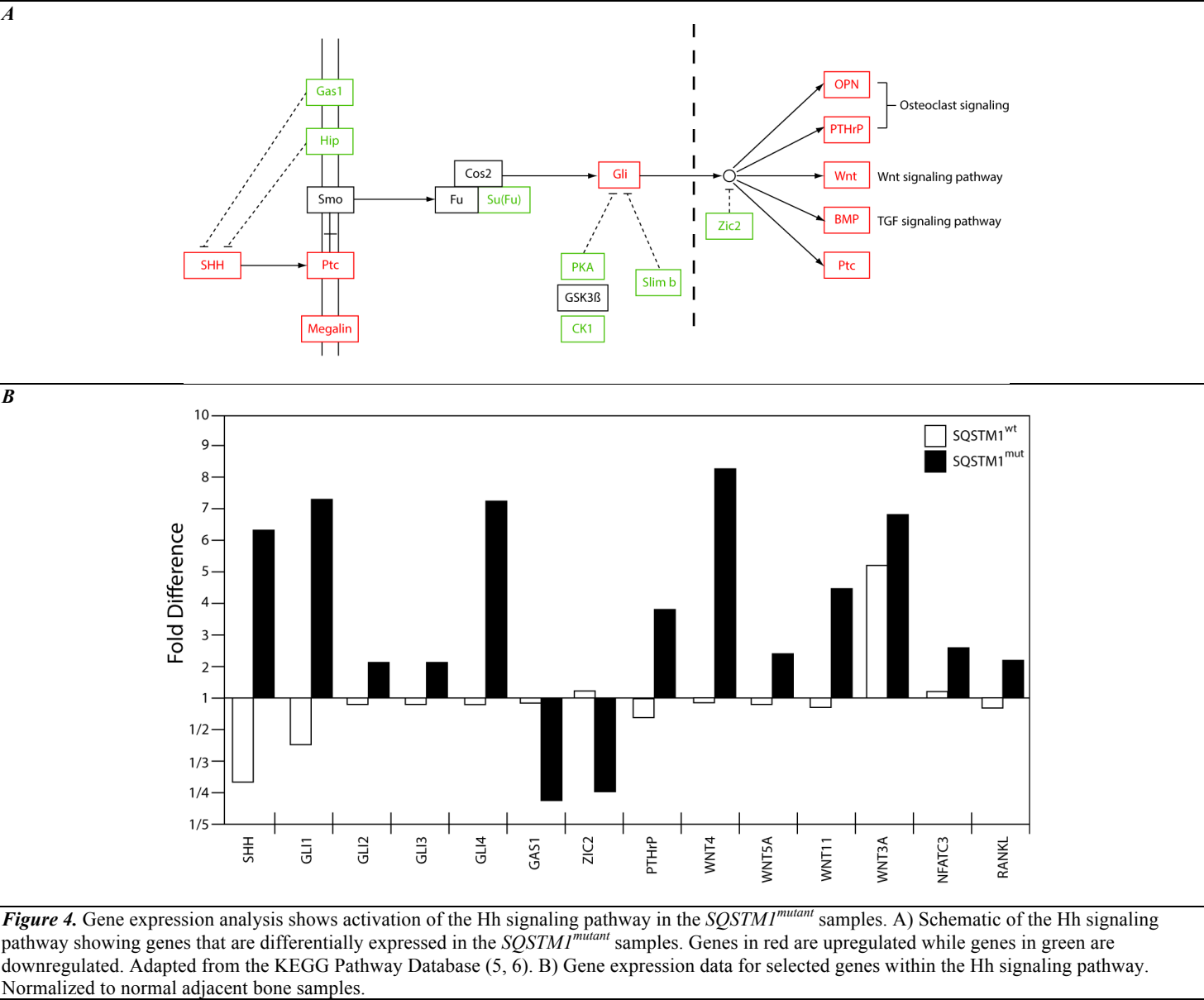


Figure 3. Gene expression analysis shows activation of the MyD88-dependent TLR signaling pathway in the *SQSTM1*^{mutant} samples. **A)** Schematic of the TLR signaling pathway showing genes that are differentially expressed in the *SQSTM1*^{mutant} samples. Genes in red are upregulated while genes in green are downregulated. Adapted from the KEGG Pathway Database (5, 6). **B)** Gene expression data for selected genes within the *MyD88*-dependent TLR signaling pathway. Gene expression normalized to normal adjacent bone samples.

Global expression analysis revealed genes downstream of the Hedgehog signaling pathway were upregulated in samples with *SQSTM1* mutations: The other signaling pathway that seems to be altered in *SQSTM1*^{mutant} samples was the Hedgehog (Hh) signaling pathway (Figure 5). Again, as in the TLR signaling pathway, specific elements of the Hh signaling pathway showed increased expression in the *SQSTM1*^{mutant} samples. These included *SHH*, *PTC2*, *GLI1*, *GLI2*, *GLI3* and *GLI4* as well as the genes that are downstream of Hh signaling including *PTHrP*, *BMPs*, and elements of the noncanonical Wnt signaling pathway (Figure 4). Inhibitors of the Hh signaling pathway were downregulated in expression in the *SQSTM1*^{mutant} samples including *GAS1*, *HHIP*, *Su(Fu)* and *ZIC2*.



Statement of Work Specific Aim 1, Part II: Allele-specific in situ PCR amplification to identify cells carrying a mutant allele of *SQSTM1*. Technical difficulties with the in situ PCR prevented us from completing this work. We were unable to consistently differentially amplify the *in situ* allelic discrimination assay. This work will continue after the grant. However, this did not hinder the other parts of the proposal.

Specific Aim 2. To determine whether a subset of cells containing the *SQSTM1* mutation can affect the overall nature of bone cell cultures containing cells with and without the mutations.

Discovery of an OB cell line carrying a *SQSTM1* mutation and comparison of expression with OB cells carrying wildtype *SQSTM1*: Although not in the original Statement of Work, we were fortunate in discovering that Menaa *et al.* had isolated and immortalized a marrow stromal cell line from a pagetic lesion (PSV10) (7). They showed that RANKL mRNA was increased in PSV10 cells compared to a normal stromal cell line or normal marrow from uninvolved bones from PDB patients (7). We obtained the PSV10 cell line from Dr. G. David Roodman (Indiana University School of Medicine) and tested the cells for a *SQSTM1* mutation. As shown in Figure 5, the PSV10 cell line has a heterozygous *SQSTM1* P387L mutation. This mutation has been previously reported in PDB families (8-11) and appears to behave similarly to other *SQSTM1* mutations described in PDB (12). We confirmed by qRT-PCR that the PSV10 cell line was of OB origin and expressed OB lineage-specific genes *BGLAP*, *COL1A1* and *RUNX2* (data not shown).

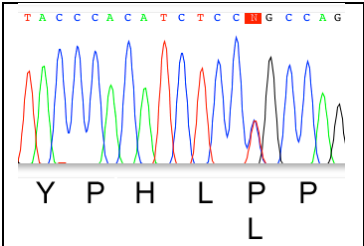


Figure 5. Heterozygous *SQSTM1*^{P387L} mutation in the PSV10 cell line.

We next tested the expression of chemokine genes as well as genes in the TLR and Hh signaling pathways in the PSV10 cell line and compared their expression to that found in an OB cell line with wildtype *SQSTM1*. hFOB1.19 (hFOB, ATCC) is an immortalized pre-OB cell line that carries a temperature sensitive mutation (tsA58) of SV40 Large T-antigen that is permissive for proliferation at 33.5°C. When the culture temperature is raised to the restrictive temperature of 39.5°C, the cells differentiate along the OB lineage and mineralize in approximately 14 days (13). We grew the PSV10 and hFOB cells with and without 1,25-(OH)₂D₃ and assayed the cells for expression of a variety of genes in the TLR and Hh signaling pathways by quantitative RealTime PCR (qRT PCR) as well as by PCR arrays (SA Biogen SuperArrays). The results are shown in Figure 6. Following stimulation by 1,25-(OH)₂D₃, we found that PSV10 showed upregulation of many of the same genes that had also been shown to be upregulated in our analysis of the *SQSTM1*^{P392L} PDB bone samples.

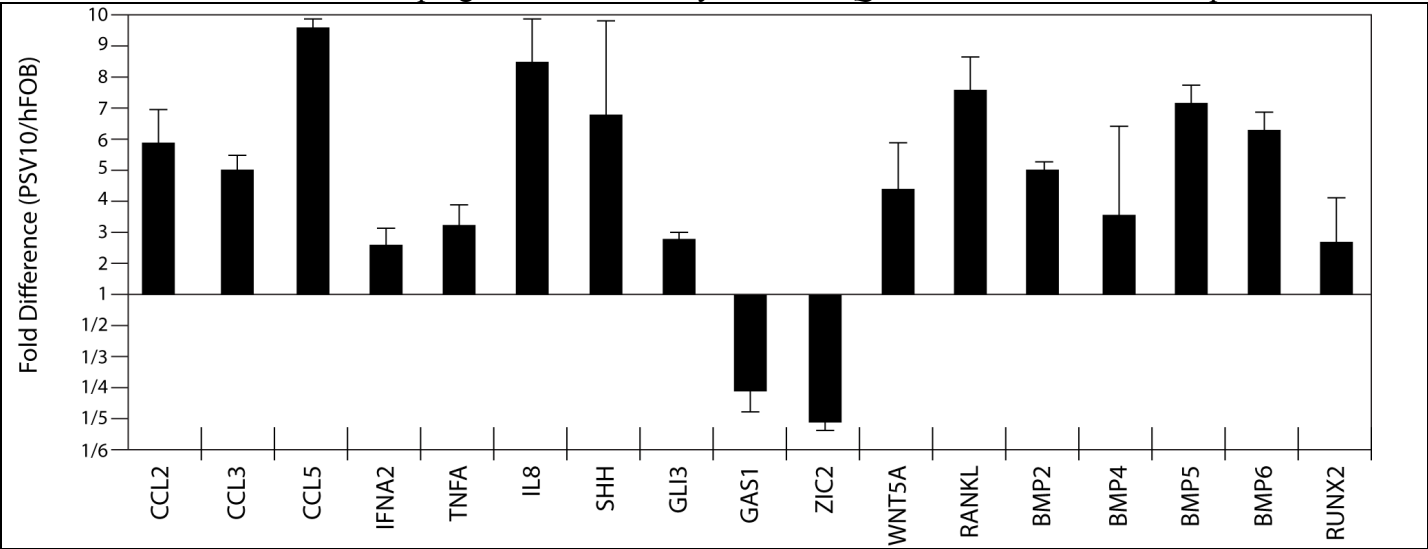


Figure 6. Comparison of expression between PSV10 and hFOB when stimulated with 1,25-(OH)₂D₃.

Differential activation of the TLR signaling pathway by

LPS: LPS has been shown to activate the MyD88-dependent TLR signaling pathway in OB and other cell types. When we stimulated PSV10 and hFOB cells with LPS and 1,25-(OH)₂D₃, we found that there was a significant difference in stimulation of *RUNX2* and *SHH* in the PSV10 cells relative to the hFOB cells as measured by qRT-PCR and this stimulation appeared to be largely due to LPS (Figure 7).

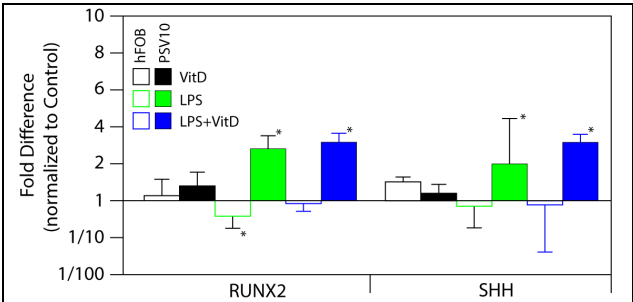
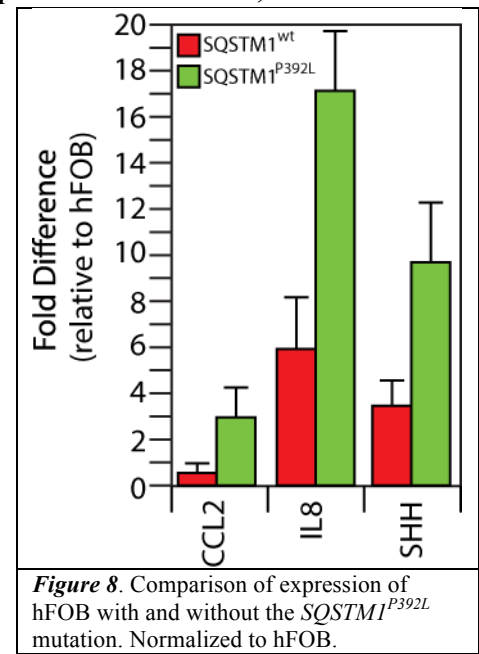


Figure 7. Comparison of the effect of LPS and VitD on expression in PSV10 and hFOB. Control is unstimulated cells.

In the original Statement of Work, we proposed to introduce the mutant SQSTM1 allele into normal human osteoblasts and to study the effect of this introduced allele on gene expression. To do this, we introduced the P392L mutation into the full-length human *SQSTM1* cDNA clone (GenBank ID: BC001874.1, IMAGE:3535436) by PCR mutagenesis and then cloned both the mutant and wildtype *SQSTM1* cDNA into a lentiviral vector under the control of the constitutive EF1 α promoter (pPS-EF1-LCS-T2A; Systems Biosciences). The incorporation of a “self-cleaving” T2A followed by an EGFP fluorescent marker allowed us to monitor transfection efficiency by immunofluorescence or by FACS analysis in the UCHC Flow Cytometry Core Facility. We transiently transfected the mutant and wildtype *SQSTM1* clones into hFOB cells and treated the cells with LPS and 1,25-(OH) $_2$ D $_3$ and assayed for expression of the chemokines that we had found to be upregulated in the PSV10 cell line by qRT-PCR. As shown in Figure 8, we were able to show increased expression of the same genes in the TLR and Hh signaling pathways in the *SQSTM1*^{P392L}-transfected hFOB cells that were seen in the *SQSTM1*^{P392L} pagetic bone and in the PSV10 cell line.

We have now developed a stable transfectant line carrying the *SQSTM1*^{P392L} mutation by repeating the transfections but also co-transfecting with a lentivirus vector carrying a puromycin resistance gene. Stable transfectants for both the *SQSTM1*^{P392L} and the puromycin resistance gene were established by maintaining the cells on G418 and puromycin.

We were not able to complete the second part of the aim, which was to assess the effect of variable ratios of cells expressing *SQSTM1* mutation on osteoclast phenotype in admixed cultures. We simply ran out of time to complete this work. We have now applied for further funding to continue this work. We also hope to use the remaining funds from this grant to complete the validation of the results and to publish the work thus far accomplished.



Key Research Accomplishments

1. Demonstration that *SQSTM1* mutations occurred somatically in the affected pagetic lesions.
2. Demonstration that the cells that contained the *SQSTM1* mutations were of osteoblastic origin rather than osteoclastic origin.
3. Demonstration that expression patterns in the affected pagetic bone differed depending on the presence of somatic *SQSTM1* mutations.
4. Demonstration that pagetic bone with *SQSTM1* mutations upregulated expression of chemokine genes important in communicating between osteoblast and osteoclast including *CCL2* (MCP1), *CCL3* (MIP1A), *CCL5* (RANTES), *CCL20* (MIP3A) as well as *CXCL6*, a gene implicated in inflammation and wound healing.
5. Demonstration that pagetic bone with *SQSTM1* mutations upregulated expression of genes in the MyD88-dependent Toll-like Receptor signaling pathway.
6. Demonstration that pagetic bone with *SQSTM1* mutations upregulated expression of genes in the Hedgehog signaling pathway.
7. Discovery that an osteoblastic cell line (PSV10) derived from a PDB patient had a P387L mutation in the *SQSTM1* gene that had been previously reported in PDB families.
8. Demonstration that PSV10 cells had similar patterns of expression to those found in patient samples of pagetic bone with *SQSTM1* mutations.
9. Demonstration that LPS induced a differential activation of gene expression in the PSV10 when compared to a normal osteoblast cell line.
10. Transiently introduced the *SQSTM1*^{P392L} mutation into normal human osteoblasts and demonstrated that the cells containing the *SQSTM1* mutation had an alteration in gene expression that was similar to both the PSV10 cell line and the pagetic bone containing the somatic *SQSTM1* mutation.
11. Establishment of a stable osteoblast cell line expressing the introduced *SQSTM1*^{P392L} mutation.
12. Demonstration of a critical role for *SQSTM1* in regulating chemokine expression through *MyD88*-mediated control of both TLR and Hh signaling pathways. This demonstrates that the mutations in *SQSTM1* found in PDB alter signaling in these pathways and lead to the initiation and/or progression of the disease.

Reportable Outcomes

- Development and characterization of osteoblastic cell lines that express the mutant form of *SQSTM1*.
- Collection and annotation of microarray and qRT-PCR data that will be submitted to the GEO database.
- Submission of applications to the FY 2012 CDMRP PRMRP program (PR120455) as well as the NIH (AR061268) based on these findings.
- We are presently completing a manuscript outlining these results and will use the remaining funds from the grant to complete the validation of the results and for page costs for the submitted publication.

Conclusion

We have used laser capture microdissection, microarray and quantitative RealTime PCR to analyze the effect of *SQSTM1* mutations in Paget's disease of bone. All evidence suggests to a critical role for *SQSTM1* in regulating *MyD88*-mediated control of both TLR and Hh signaling pathways and that mutations in *SQSTM1* found in PDB alter chemokine signaling between the osteoblast and the osteoclast. The significance of this work is that it is a fundamentally new paradigm for *SQSTM1*-mediated PDB, which places the osteoblast cell squarely in the center of the disease's etiology. This discovery has generated a host of new hypotheses to test including the mechanism by which mutations in *SQSTM1* result in the upregulation of these signaling pathways. It opens a completely new direction in PDB research that could provide new targets for therapeutic intervention that are better than the current bisphosphonate therapy. It also provides new insights into bone remodeling and the regulation of the interactions between osteoblast and osteoclast. In short, this revolutionizes the field of Paget's disease research.

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Appendices

None